

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Diverse Responses of Autoantibodies to the Angiotensin II Type 1 Receptor in Primary Aldosteronism

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1717650> since 2019-11-25T23:36:33Z

Published version:

DOI:10.1161/HYPERTENSIONAHA.119.13156

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

**Diverse responses of autoantibodies to the angiotensin II type 1 receptor
in primary aldosteronism**

Tracy Ann Williams^{1,2}, Diana Jaquin¹, Jacopo Burrello², Aurélie Philippe³, Yuhong Yang¹,

Petra Rank¹, Nina Nirschl¹, Lisa Sturm¹, Christoph Hübener⁴, Duska Dragun^{3,5},

Martin Bidlingmaier¹, Felix Beuschlein^{1,6}, Martin Reincke¹

¹Medizinische Klinik und Poliklinik IV, Klinikum der Universität, Ludwig-Maximilians-Universität München, Munich, Germany

²Division of Internal Medicine and Hypertension, Department of Medical Sciences, University of Turin, Turin, Italy

³Clinic for Nephrology and Critical Care Medicine, Campus Virchow-Klinikum and Centre for Cardiovascular Research, Medical Faculty of the Charité Berlin, Berlin, Germany

⁴Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, Klinikum der Universität München, Munich, Germany

⁵ Berlin Institute of Health, Anna-Luisa-Karsch Str. 2 10178 Berlin, Germany

⁶Klinik für Endokrinologie, Diabetologie und Klinische Ernährung, Universitätsspital Zürich, Zürich, Switzerland

Corresponding author: Tracy Ann Williams, Medizinische Klinik und Poliklinik IV, Klinikum der Universität München, LMU München, Ziemssenstr. 1, D-80336 München, Germany
Tel: +49 89 4400 52941; Fax: +49 89 4400 54428
Email: Tracy.Williams@med.uni-muenchen.de

Total words: 4,200 + 4 tables + 1 colour figure

Online supplement: 3 figures + 1 table

Running title: Angiotensin II type 1 receptor autoantibodies

Key words: aldosterone-producing adenoma, bilateral adrenal hyperplasia, preeclampsia, primary hypertension

1 **Abstract**

2 Primary aldosteronism (PA) is a common form of endocrine hypertension mainly caused by a
3 unilateral aldosterone-producing adenoma (APA) or bilateral adrenal hyperplasia (BAH).
4 Autoantibodies that activate the angiotensin II type 1 receptor (AT1R-Abs) have been reported in
5 patients with disorders associated with hypertension. Our objective was to assess AT1R-Ab levels
6 in patients with PA (APA, n=40; BAH, n=40) relative to patients with primary hypertension (n=40),
7 preeclampsia (n=23) and normotensive individuals (n=25). AT1R-Abs in whole sera were measured
8 using 2 different ELISAs which gave contrasting results. A functional cell-based assay was used to
9 quantify activation of the angiotensin II type 1 receptor (AT1R) using whole sera or affinity-purified
10 antibodies in the absence or presence of losartan (a specific AT1R antagonist). Serum samples
11 from all groups displayed different levels of AT1R activation with different responses to losartan.
12 Patients with BAH displayed higher losartan-independent affinity-isolated agonistic AT1R-Ab levels
13 compared with patients with APA ($P<0.01$) and with normotensive individuals ($P<0.0001$). In
14 patients with APA, BAH and PH combined, higher aldosterone-to-renin ratios and lower plasma
15 renin concentrations were associated with higher compared with lower agonistic AT1R-Abs levels.
16 In patients with PA, higher AT1R-Ab activity was associated with an increased likelihood of a
17 diagnosis of BAH compared with APA and with the presence of adrenal hyperplasia detected by
18 computed tomography. Taken together these data suggest that agonistic AT1R-Abs may have a
19 functional role in a subgroup of patients with primary aldosteronism.

20 **Introduction**

21 Primary aldosteronism (PA) is a form of endocrine hypertension caused by the overproduction of
22 aldosterone from one or both adrenal glands (unilateral or bilateral PA, respectively). Unilateral PA
23 is predominantly caused by an aldosterone-producing adenoma (APA) and bilateral forms by
24 bilateral adrenocortical hyperplasia (BAH).¹ APA and BAH mainly arise sporadically but uncommon
25 familial forms have been described (familial hyperaldosteronism types I-IV).^{2,3} Substantial progress
26 has been made in understanding the pathophysiology of familial PA and sporadic APAs with the
27 identification of germline mutations causing 4 familial forms of hyperaldosteronism⁴⁻⁹ and somatic
28 mutations which drive aldosterone excess in 50-80% of APAs.^{2,10-12} These advances, however, have
29 not been replicated in understanding the pathogenesis of sporadic BAH. The bilateral nature of
30 the disease led to the proposal of circulating factors, which could trigger bilateral chronic
31 stimulation of the adrenal *zona glomerulosa*.

32
33 Graves disease is an established example of an autoimmune disease caused by agonistic
34 autoantibodies which activate the thyroid stimulating hormone receptor (TSHR) resulting in
35 thyroid hormone production and thyroid cell proliferation.¹³⁻¹⁵ In addition to agonistic antibodies,
36 antagonistic and neutral autoantibodies to the TSHR have been described which either block TSH
37 activity or have no apparent effect.¹⁵ Autoimmune responses to other G protein coupled receptors
38 have been reported in several studies implicating a role for autoantibody activation of the
39 angiotensin II type 1 receptor (AT1R), the α_1 -adrenergic and β_1 -adrenergic receptors in several
40 cardiovascular disorders.¹⁶⁻²⁵ Furthermore, multiple studies have reported the detection of
41 autoantibodies to the angiotensin II type 1 receptor (AT1R-Abs) in patients with preeclampsia.^{20,26}
42 AT1R-Abs which recognize the AFHYESQ peptide (position 165-171) in the second extracellular
43 loop of the AT1R have been implicated as autoantibody-mediated drivers of AT1R agonism.

Specifically, ELISAs employing an immobilized synthetic AFHYESQ peptide are often used to assay AT1R-Ab levels.^{20,27} Using either ELISA or functional assays, AT1R-Abs have also been reported in patients with PA in whom AT1R-Ab levels are variously reported as higher in patients with APA than with BAH, higher in BAH compared with APA or similar levels in both subtypes of PA.²⁸⁻³⁰ These studies have either used ELISA-based assays, which do not provide information on the agonistic effect of AT1R-Abs, or have included only small cohorts of patients with PA.

Our objective was to establish if functionally active AT1R-Abs are present in a large cohort of 80 patients with PA (40 patients with APA, 40 with BAH) in comparison with primary hypertension (PH, n=40), preeclampsia (PE, n=23) and normotensive individuals (NT, n=25) using 3 assays: 2 different ELISA-based assays both using immobilized full-length AT1R and a highly sensitive cell-based AT1R activation functional assay.

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Methods

Patient samples

For quantification of AT1R-Abs and AT1R activating activity, serum samples from 80 patients with PA (40 with APA and 40 with BAH), 40 with primary hypertension (PH), 23 women with preeclampsia (PE) and 25 normotensive blood donors (NT) were used. PA was diagnosed in accordance with the Endocrine Society guideline.³¹ Patients were screened for PA using the plasma aldosterone-to-direct renin concentration ratio and diagnosis was confirmed by the intravenous saline load test according to local criteria.³² All patients with confirmed PA underwent computed tomography (CT) scanning and adrenal venous sampling. The cut-off selectivity index to

determine success of catheterization was ≥ 2 and for the lateralization index to diagnose unilateral PA ≥ 4 .³² PH was diagnosed in accordance with the ESH/cardiology guidelines³³ after ruling out PA, pheochromocytoma and Cushing syndrome. PE and Graves disease were diagnosed as described previously.^{34,35} Blood sampling for patients with PA and PH was performed at screening for secondary hypertension. Whenever possible, patients were under no treatment or before the beginning of an anti-hypertensive therapy. When necessary blood pressure was controlled using the calcium channel blocker verapamil or the α -blocker doxazosin, alone or in combination, in accordance with the ES guideline.³¹ Blood samples from patients with Graves disease were withdrawn at the first medical visit and from patients with PE in the third trimester. All participants gave written informed consent and the protocol was approved by the local ethics committee.

79

80 ***AT1R autoantibody measurements***

All AT1R-Abs were measured using 3 different assays. Two commercially available ELISA kits were used to quantify autoantibodies against the recombinant human full-length AT1R (ELISA-Creative Diagnostics and ELISA-CellTrend).^{36, 37} The third assay was a cell based AT1R activation assay (Invitrogen Gene BLazer beta-lactamase reporter system) to measure agonistic AT1R activity in total serum and in affinity-isolated IgG fractions after pre-incubation for 1 hour with vehicle or 100 μ M losartan. Immunoglobulins (IgGs) were affinity isolated on protein A/G agarose from 1 mL patient serum and 1/10 of the affinity-isolated IgGs was used in the functional assay. The isolation of IgGs on protein A/G agarose and their depletion from serum samples was confirmed by Western blotting using a horseradish peroxidase conjugated goat anti-human antibody (Millipore, 1:50000 dilution) (**Figure S1**).

91

92 The cell based AT1R activation assay employed AT1R-*bla* U2OS cells which stably express the AT1R
93 linked at the C-terminus to the Gal4-VP16 transcription factor via a TEV (Tobacco Etch Virus)
94 protease cleavage site (E-X-X-Y-X-Q-G/S) (Invitrogen). The U2OS cells also stably express TEV
95 protease-tagged- β -arrestin/TEV and a β -lactamase reporter gene with Gal4-responsive upstream
96 activator sequences. Following AT1R activation, the TEV-protease- β -arrestin is recruited to the
97 AT1R receptor C-terminus and cleaves the TEV cleavage sequence releasing GAL4-VP16 which
98 activates expression of the β -lactamase reporter gene. A Förster resonance energy transfer (FRET)
99 substrate comprising coumarin and fluorescein fluorophores was used to measure reporter gene
100 activity (ThermoFisher, LiveBLAzer-FRET B/G substrate). In the absence of β -lactamase reporter
101 gene expression, the FRET substrate is intact, coumarin excitation transfers fluorescence
102 resonance energy to fluorescein resulting in emission of green fluorescence. When the substrate is
103 cleaved, energy transfer is disrupted and a blue fluorescence signal is emitted from coumarin
104 excitation. Reporter activities, corresponding to AT1R activation, are given as response ratios
105 which are the ratio of coumarin to fluorescein fluorescence signals (ratio of cleaved to uncleaved
106 substrate) normalized for negative control wells (mock-treated cells).

107

108 ***TSHR activation assay***

109 Activity of affinity-isolated IgGs from serum of Graves disease patients was measured using a TSHR
110 agonistic cell-based assay to determine if autoantibody functional activity was maintained
111 following the IgG isolation procedure. The assay uses TSHR ACTOne cells, a HEK-293 CNG (human
112 embryonic kidney-293 cyclic nucleotide gated) cell line with overexpression of recombinant
113 human TSHR (MyBiosource). The modified CNG channel opens in response to elevated
114 intracellular cAMP levels and the resultant ion influx and membrane depolarization is measured
115 with a fluorescent membrane potential dye. The assay measures intracellular cAMP levels as a

116 response ratio between TSHR ACTOne cells compared with the parental control cell line (HEK-293
117 CNG cells).

118

119 ***Adrenal morphology***

120 CT imaging was used to classify absence or presence of adrenal hyperplasia in adrenals with an
121 abnormal morphology. The absence of hyperplasia group included adrenals with an adenoma but
122 without hyperplasia, the presence of hyperplasia group included adrenals with hyperplasia alone
123 or hyperplasia and an adenoma. Hyperplasia was defined as mean limb width ≥ 5 mm.³⁸ Patients
124 with no adrenal abnormality visible on CT images were excluded from the morphologic analysis.

125

126 ***Statistical analyses***

127 Data were analyzed with the Kolmogorov-Smirnov and Shapiro-Wilk tests to determine
128 distributions. Group differences were calculated for normally distributed data using the ANOVA
129 and post-hoc Bonferroni tests. Non-normally distributed data were analyzed using the Kruskal-
130 Wallis test. Accordingly, data are expressed as mean \pm SD or median (25th to 95th percentile).
131 Categorical variables are presented as absolute numbers and percentages and differences were
132 analyzed with a Chi-square test. Adjusted logistic regression analyses were performed to assess
133 associations of AT1R-Abs and the diagnosis of BAH. IBM SPSS Statistics version 22.0 was used for
134 all analyses.

135

136 **Results**

137 ***Clinical parameters of patients with primary aldosteronism versus primary hypertension***

138 Groups of patients with APA and BAH had the same age as patients with PH and a similar gender
139 distribution with no significant differences in the proportion of males and females between APA,

140 BAH and PH groups (47.5-57.9%). There were no significant between-group differences in systolic
141 or diastolic blood pressure at baseline or in body mass index in patients with APA, BAH and PH
142 (**Table 1**). As expected, patients with APA or BAH had higher plasma aldosterone concentrations
143 (PAC) and lower direct plasma renin concentrations (DRC) at baseline relative to the PH group
144 (PAC: APA group, 569 [283-1071]; BAH, 416 [311-583]; PH 225 [128-394] pmol/L and DRC: APA
145 group, 4.3 [2.0-11.2]; BAH, 3.4 [2.0-7.3]; PH, 18.2 [8.9-45.1] mU/L). Likewise, patients with APA
146 had lower serum potassium concentrations compared with patients with BAH and PH (APA group,
147 2.9 [2.6-3.2]; BAH, 3.3 [3.0-3.7]; PH 3.9 [3.6-4.2] mmol/L) (**Table 1**).

148

149 ***ELISA quantification of AT₁R-Abs in different groups***

150 Autoantibodies recognizing epitopes on the full-length human recombinant AT1R in serum from
151 patients with APA, BAH, PH, PE and NT were measured using 2 different ELISAs. Using one
152 approach (ELISA-Creative Diagnostics), patients with PE displayed significantly higher AT1R-Ab
153 levels compared with all other groups ($P<0.0001$ for all comparisons). The titer of AT1R-Abs was
154 highly similar in the APA and BAH groups (APA group, [0.06-0.21]; BAH, 0.12 [0.06-0.26] ng/mL)
155 with no differences observed compared with either the PH or NT groups (PH group, 0.15 [0.10-
156 0.25]; NT, 0.11 [0.01-0.19] ng/mL) (**Figure, panel A; Table S1**). We also used a second ELISA (ELISA-
157 CellTrend) based on AT1R-Ab binding to the full-length AT1R in its native conformation.^{36, 37}
158 Patients with APA and BAH displayed highly similar levels of AT1R-Abs (APA group, 14.2 [10.4-
159 22.0]; BAH, 14.1 [10.1-19.7] U/mL) which were not significantly different from the PH or NT groups
160 (PH group, 13.5 [10.7-18.7]; NT, 11.4 [10.6-20.8] ng/mL) (**Figure, panel B, Table S1**). However,
161 AT1R-Ab levels were significantly lower in patients with PE (8.7 [6.9-11.6] ng/mL) compared with
162 all other groups ($P<0.05$ for all comparisons).

163

164 ***Quantification of AT1R agonistic activity in serum samples from different groups***

165 We tested if serum from the different subgroups of patients and individuals could activate the
166 AT1R in a cell based functional assay. Treatment of cells with angiotensin II (0-500 pM)
167 demonstrated a dose-dependent effect on AT1R activation which was ablated by pre-incubation of
168 the cells for 1 h with the AT1R antagonist losartan (100 μ M). The assay measured a specific AT1R
169 functional response to 50 pM angiotensin II which was significantly higher than a corresponding
170 incubation in the presence of losartan ($P<0.05$) (**Figure S2**). Higher AT1R agonistic activity was
171 measured in serum samples from all groups ($P<0.001$ for absence *versus* presence of losartan for
172 each group). There were no between-group differences for AT1R agonist activity in the absence of
173 losartan. However, in the presence of losartan there were overall differences in the measured
174 functional activation of the AT1R ($P<0.001$) with the BAH group showing higher activity compared
175 with the APA ($P=0.001$), PE ($P<0.0001$) and NT groups ($P<0.0001$). The PH group also displayed
176 higher levels of functional AT1R-Abs relative to the NT ($P<0.0001$) and the PE groups ($P=0.001$)
177 (**Figure, panel C, Table S1**).

178

179 ***Affinity isolation of IgG fractions from different groups of serum samples***

180 To determine if the losartan-independent AT1R activating activity in serum samples was due to
181 IgGs or to other circulating factors, such as angiotensin II, IgGs were affinity-isolated from all
182 serum samples on protein A/G-agarose to assess AT1R agonist activity in the cell based AT1R
183 functional assay (**Figure S1, Figure S2**). We first tested if the IgG affinity-isolation procedure
184 produced functionally active autoantibodies. For this, IgGs were isolated from the serum of
185 patients with Graves disease ($N=9$) and measured TSHR activation using a cell based functional
186 assay. Using IgG fractions isolated from patients with Graves disease, comparison of TSHR
187 activation in the ACT-ONE cell line (with stable overexpression of the human TSHR) with the

parental cell line (without expression of recombinant human TSHR) demonstrated that 6 of the 9 IgG fractions displayed TSHR agonistic activity (**Figure S3**). The remaining 3 IgG fractions exhibited no significant TSHR activation indicating neutral or blocking activity to the TSHR (**Figure S3**). Overall, the approach used for the affinity isolation of autoantibodies from patients with Graves disease maintained TSHR agonist functional activity thereby validating the method used for the isolation of IgG fractions.

Quantification of AT1R agonistic activity in affinity-isolated IgG fractions from different groups

There were group differences in the cell-based assay response (overall difference $P<0.001$) using affinity-isolated IgGs. The BAH, PH and PE groups displayed higher levels of AT1R activating autoantibodies compared with the NT group ($P<0.0001$, $P=0.007$ and $P<0.0001$, respectively) and the BAH group had higher functional AT1R-Ab levels than the APA group ($P=0.01$). The agonistic AT1R-Ab levels were not abolished in the presence of losartan and significant group differences were observed (**Table S1**). Higher losartan-independent AT1R functional activity was measured with IgGs isolated from patients with BAH, PH and PE compared with the NT group ($P<0.0001$, $P=0.006$ and $P=0.016$, respectively) and in the BAH *versus* APA groups ($P=0.01$) (**Figure, panel D, Table S1**). Comparison of AT1R activation in the cell assay with the functional response obtained with angiotensin II in the dose-response assay indicated that the median AT1R activation achieved with affinity-isolated antibodies from patients with BAH in the presence or absence of losartan was equivalent to 50 to 100 pM angiotensin II (**Figure S2, Table S1**).

Clinical parameters of patients according to functional AT1R-Ab levels

Affinity-purified agonistic AT1R-Ab levels were categorized into higher and lower AT1R-Ab levels according to the median AT1R-Ab activity in the cell-based assay for patients with APA, BAH and

PH combined. In this cohort, in the absence of losartan, patients with BAH had higher AT1R-Ab levels (BAH represented 41.2% of 68 patients of the combined cohort with higher AT1R-Ab levels compared with 23.1% of 52 patients with lower AT1R-Ab levels, $P=0.037$) (**Table 2**). Patients with APA had lower AT1R-Ab levels (APA represented 23.5% of 68 patients of the combined cohort with higher AT1R-Ab levels compared with 46.2% of 52 patients with lower AT1R-Ab levels, $P=0.009$) (**Table 2**). Although functional AT1R-Ab levels were similar in the BAH *versus* PH groups (**Figure, panel D; Table S1**), patients with PH with lower *versus* higher AT1R-Ab levels were similarly distributed in the combined cohort (APA + BAH + PH). The PH group with lower AT1R-Ab levels comprised 30.7% of 52 patients of the combined cohort compared with 35.3% of 68 patients with higher levels ($P=0.603$) (**Table 2**).

In the APA, BAH and PH combined cohort, higher levels of agonistic AT1R-Abs were also associated with a higher aldosterone-to-renin ratio (ARR_DRC) and a lower direct renin concentration (DRC) in the absence of losartan (DRC: 5.7 mU/mL [2.2-27.0] *versus* 11.7 mU/mL [5.7-31.8], $P=0.011$; ARR_DRC: 47 [13-139] *versus* 23 [10-55], $P=0.029$, for higher *versus* lower AT1R-Ab levels, respectively) and these differences were maintained in the presence of losartan (**Table 2**).

Patients with PA with higher agonistic AT1R-Ab levels, in the absence of losartan, had an increased likelihood of a diagnosis of BAH *versus* APA after adjustment for confounding effects of age, systolic BP, PAC or DRC (**Table 3**). Higher losartan-independent agonistic AT1R-Ab levels were not associated with a diagnosis of BAH compared with APA after correction for systolic BP and PAC. There was no association of higher AT1R-Ab levels with a diagnosis of BAH compared with PH in either the absence of presence of losartan (**Table 3**).

236 ***Adrenal morphology according to functional AT1R-Ab levels***

237 Adrenal abnormalities were absent on CT images in 3 patients diagnosed with APA and in 17
238 patients diagnosed with BAH, and these cases were excluded from the morphologic analysis.
239 Higher affinity-purified AT1R-Ab levels in the absence of losartan were associated with the
240 presence of adrenal hyperplasia when AT1R-Ab levels were treated as either a continuous variable
241 (AT1R activating activity response ratio, 0.3 [0.26-0.39] *versus* 0.26 [0.23-0.29] in the presence and
242 absence of hyperplasia, respectively, $P=0.011$) or categorized as higher or lower according to the
243 median AT1R-Ab level (76.0 % of 25 patients with adrenal hyperplasia had higher AT1R-Ab levels
244 compared with 37.1% of 35 patients without adrenal hyperplasia, $P=0.003$) (**Table 4**). In the
245 presence of losartan, AT1R-Ab activities were similar in the presence *versus* absence of
246 hyperplasia groups (**Table 4**).

247

248 The distribution of individual patients with PA (APA and BAH) with adrenal hyperplasia according
249 to AT1R-Ab activating activity is shown in **Figure, panel D**. In patients with PA, 83.3% of 12 and
250 69.2% of 13 patients of patients classified with adrenal hyperplasia in the APA and BAH groups,
251 respectively, had AT1R-Ab levels above or equal to the median value for their group in the
252 absence of losartan (**Figure, panel D**).

253

254 **Discussion**

255 Autoantibodies that potentially elicit a functional response by binding to G protein-coupled
256 receptors have been described in several cardiovascular disorders.²⁵ Many studies have reported
257 AT1R-Ab binding to an epitope in the second extracellular loop (AFHYESQ) of the AT1R in different
258 groups of patients.²⁰ The best characterized is AT1R-Abs in PE where a functional role has been
259 implicated using cardiomyocyte contraction assays in which assay response was ablated either by

260 the AT1R antagonist losartan or with the AFHYESQ peptide.^{20,39} The prevalence of AT1R-Abs in PE
261 varies widely with reports employing an ELISA ranging from 48% of 58 patients⁴⁰ to 100% of 25
262 patients.²⁰ However, targeting the AFHYESQ peptide in ELISA assays has limitations because
263 binding to linear immobilized peptides may not correlate with AT1R agonism and binding to
264 conformational epitopes cannot be assessed.⁴¹ A commercially available ELISA (ELISA-CellTrend),
265 routinely used in solid organ transplantation, has been developed based on autoantibody binding
266 to the full-length AT1R in the native conformation.³⁷ Using this conformation sensitive assay, we
267 demonstrated highly contrasting low AT1R-Ab levels compared with a different ELISA method
268 which appears to greatly overestimate the level of AT1R-Abs in patients with preeclampsia.

269
270 The pathophysiology of sporadic BAH is poorly understood. Advances in knowledge are hampered
271 by the highly limited availability of tissue samples for molecular studies because patients with BAH
272 are not usually surgically-treated. Despite this, recent studies have suggested a role for
273 adrenocortical hyperplasia in patients with bilateral but asymmetrical inappropriate aldosterone
274 production⁴² or a role for small clusters of cells located beneath the adrenal capsule with high
275 aldosterone synthase expression (called aldosterone-producing cell clusters) in surgically-treated
276 patients diagnosed with bilateral PA.⁴³ Thus, BAH may not be a distinct entity but a disorder
277 comprising clinical and biochemical variability arising from morphological heterogeneity
278 representing the variable response of the adrenal cortex to circulating, environmental and genetic
279 factors.

280
281 A role for autoantibodies that trigger bilateral chronic stimulation of the adrenal *zona glomerulosa*
282 via activation of the AT1R has been proposed⁴⁴ but a pathogenic role for AT1R-Abs in PA remains
283 unclear because of conflicting reports that used different methods for assessment of antibody

284 levels.²⁸⁻³⁰ One study found a 2-fold increase of AT1R-Abs against the AFHYESQ peptide in an ELISA
285 in patients with APA (n=26) compared with patients with BAH (n=20) and proposed the use of this
286 assay as a potential diagnostic tool to differentiate the two different types of PA.²⁸ Using a similar
287 ELISA-based AFHYESQ assay no difference in AT1R-Ab levels were observed in 44 patients with PA
288 (15 with APA, 29 with BAH) compared with 18 normotensive individuals (n=18) and no difference
289 in AT1R-Ab levels between the patients with APA and BAH.³⁰ However, measuring antibody
290 binding to the linear AFHYESQ peptide in ELISA assays, as used in many studies, does not
291 necessarily correlate with AT1R agonism.

292
293 To address the agonistic activity of AT1R-Abs in PA, Kem et al²⁹ reported increased AT1R-Ab levels
294 in patients with PA (n=13) compared with control subjects (n=20) using cell-based assays to
295 measure a functional response in AT1R-transfected cells and reported the contractile effects of
296 the isolated IgGs in perfused rat cremaster arterioles. In contrast to other reports, an increased
297 prevalence of AT1R-Abs in patients with BAH relative to patients with APA was reported.²⁹
298 However, the number of patients with PA assessed for AT1R-Ab levels was small, the stimulating
299 activity of low potency and the affinity-isolated antibodies did not elicit a dose-dependent
300 functional effect.²⁹

301
302 The diverse observations for the prevalence and potential role of AT1R-Abs and the limited
303 understanding of the pathogenesis of bilateral PA highlight the need for studies to measure
304 autoantibodies using robust functional assays in large and well characterized cohorts of patients
305 with PA. Herein, we assessed AT1R-Ab levels in a cohort of 80 patients with PA diagnosed in
306 accordance with rigorous criteria and with subtype diagnosis (APA *versus* BAH) defined by adrenal
307 venous sampling. Following this approach, ELISA-based measurements using the immobilized full-

length AT1R gave contrasting results for AT1R-Ab levels in patients with PE and did not reveal statistical differences between patients with BAH or APA compared with PH or NT. We hence also used a cell-based AT1R functional assay which exploits specific activation of the β -lactamase reporter gene upon ligand binding to the AT1R. With this assay, similar levels of AT1R activation were measured in whole serum from all groups. However, between-group differences were shown using affinity-isolated IgGs which demonstrated significantly higher levels of agonistic AT1R-Abs in patients with BAH compared with APA and in patients with BAH, PH and PE relative to the NT group both in the presence and absence of losartan.

These activities implicate the existence of an alternative epitope structurally remote from losartan binding sites. AT1R is increasingly recognized as a multi-ligand binding surface and epitopes discovered in solid organ transplant patients are not identical with those in patients with PE³⁷. Some reports suggest that, in addition to classical G protein-mediated signaling, “biased” AT1R signaling mediated by β -arrestin^{45,46} may play a role in aldosterone production and have pathological implications for the progression to heart failure after myocardial infarction.^{47,48} Because losartan antagonizes G protein signaling but is ineffective in ablating β -arrestin-mediated signaling,^{47,48} the losartan-independent activity we report presumably comprises “biased” AT1R signaling.

We also demonstrate that higher agonistic AT1R-Ab levels are associated with clinical parameters characteristic of autonomous aldosterone production in PA such as higher aldosterone-to-renin ratios and lower plasma renin levels. The degree of functional activity of AT1R-Abs in this study appears low but is potentially pathologically relevant because the median AT1R-Ab agonistic activity in patients with BAH corresponds to greater than that achieved with 50 pM angiotensin II,

332 a concentration similar to plasma angiotensin II concentrations reported in patients with chronic
333 kidney disease and considerably higher than in healthy individuals.⁴⁹

334
335 A potential pathogenic role of agonistic AT1R-Abs in PA is suggested by the association of higher
336 active AT1R-Ab levels - in the absence but not in the presence of losartan - with an increased
337 likelihood of a diagnosis of BAH compared with APA and with an increased incidence of adrenal
338 hyperplasia. Adrenals harboring an APA also often display focal or diffuse cortical hyperplasia
339 adjacent to the adenoma.^{42,50} It is notable that within the group of patients with APA, those with
340 evidence of hyperplasia at CT scanning tend to display higher levels of AT1R-Ab agonistic activity
341 compared with patients with APA without hyperplasia. The imaging data should however be
342 treated with caution considering the potential for incorrect classification of an adenoma *versus*
343 hyperplasia.

344
345 Taken together the present data indicate that AT1R-Abs may play a role in patients with BAH
346 which could feasibly exacerbate the effects of additional pathophysiological factors such as
347 aldosterone-producing cell clusters which have been reported as larger, more numerous and with
348 a higher prevalence of aldosterone-driver mutations than normal adrenals.⁴³ Notwithstanding the
349 observations reported herein, the possibility that AT1R-Abs are a marker of hypertension rather
350 than having a pathogenic role cannot be excluded.

351
352 In conclusion, some patients with disorders related to hypertension have activating
353 autoantibodies to the AT1R. Some AT1R-Abs function via a mechanism diverse from the classical G
354 protein-mediated AT1R signaling and implicate a role for losartan-independent “biased” AT1R

355 signaling. Overall, the present study suggests a role for agonistic autoantibodies to the AT1R in a
356 subgroup of patients with PA, comprising those patients with adrenal hyperplasia,

357

358 **Perspectives**

359 A role for AT1R-Abs has been implicated in several cardiovascular disorders but evidence for a
360 direct function in disease pathophysiology is lacking. *In vivo* experiments in mice subjected to
361 infusion of AT1R-Abs from patients with PA could clarify the impact of AT1R-Abs on aldosterone
362 production. A longitudinal analysis is planned to measure the response of AT1R-Ab levels to
363 adrenal surgery or mineralocorticoid receptor antagonism in patients with PA with long term
364 follow up. Epitope mapping using synthetic peptides to competitively abolish autoantibody-
365 mediated AT1R activation will aid the identification of AT1R-Ab binding sites and establish any role
366 for autoantibodies in “biased” signaling.

367

368 **Sources of Funding**

369 This work was supported by the European Research Council (ERC) under the European Union’s
370 Horizon 2020 research and innovation programme (grant agreement No [694913] to M Reincke)
371 and by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation)
372 Projektnummer: 314061271-TRR 205 to F Beuschlein, M Reincke, TA Williams; and by DFG grants
373 BE 2177/13-1 and BE 2177/18-1 to F Beuschlein and RE 752/20-1 to M Reincke. This work was also
374 supported by the Else Kröner-Fresenius Stiftung in support of the German Conns Registry-Else-
375 Kröner Hyperaldosteronism Registry (2013_A182 and 2015_A171 to M Reincke) and from a grant
376 from the Ministero dell’Istruzione, dell’Università e della Ricerca (MIUR, ex-60% 2015-2016 to TA
377 Williams).

378

379 **Conflicts of Interest Disclosure**

380 None

381

382 **References**

383 1) Stowasser M, Gordon RD. Primary aldosteronism: changing definitions and new concepts of
384 physiology and pathophysiology both inside and outside the kidney. *Physiol Rev.* 2016;96:1327-
385 1384. doi: 10.1152/physrev.00026.2015.

386 2) Prada ETA, Burrello J, Reincke M, Williams TA. Old and new concepts in the molecular
387 pathogenesis of primary aldosteronism. *Hypertension* 2017;70:875-881.
388 doi: 10.1161/HYPERTENSIONAHA.117.10111.

389 3) Young WF Jr. Diagnosis and treatment of primary aldosteronism: practical clinical perspectives.
390 *J Intern Med.* 2019;285:126-148. doi: 10.1111/joim.12831.

391 4) Lifton RP, Dluhy RG, Powers M, Rich GM, Cook S, Ulick S, Lalouel JM. A chimaeric 11 beta-
392 hydroxylase/aldosterone synthase gene causes glucocorticoid-remediable aldosteronism and
393 human hypertension. *Nature* 1992;355:262-265

394 5) Choi M, Scholl UI, Yue P, et al. K⁺ channel mutations in adrenal aldosterone-producing
395 adenomas and hereditary hypertension. *Science* 2011;331:768-772. doi:
396 10.1126/science.1198785.

397 6) Scholl UI, Stölting G, Nelson-Williams C et al. Recurrent gain of function mutation in calcium
398 channel CACNA1H causes early-onset hypertension with primary aldosteronism. *Elife*
399 2015;24;4:e06315. doi: 10.7554/eLife.06315.

400 7) Scholl UI, Stölting G, Schewe J, et al. CLCN2 chloride channel mutations in familial
401 hyperaldosteronism type II. *Nat Genet.* 2018;50:349-354. doi: 10.1038/s41588-018-0048-5.

402 8) Fernandes-Rosa FL, Daniil G, Orozco IJ, Göppner C, El Zein R, Jain V, Boulkroun S, Jeunemaitre X,
403 Amar L, Lefebvre H, Schwarzmayer T, Strom TM, Jentsch TJ, Zennaro MC. A gain-of-function
404 mutation in the CLCN2 chloride channel gene causes primary aldosteronism. *Nat Genet.*
405 2018;50:355-361. doi: 10.1038/s41588-018-0053-8.

406 9) Perez-Rivas LG, Williams TA, Reincke M. Inherited forms of primary hyperaldosteronism: new
407 genes, new phenotypes and proposition of a new classification. *Exp Clin Endocrinol Diabetes*
408 2019;127:93-99. doi: 10.1055/a-0713-0629.

409 10) Williams TA, Monticone S, Schack VR et al. Somatic ATP1A1, ATP2B3, and KCNJ5 mutations in
410 aldosterone-producing adenomas. *Hypertension* 2014;63:188-195.
411 doi: 10.1161/HYPERTENSIONAHA.113.01733.

412 11) Fernandes-Rosa FL, Williams TA, Riester A et al. Genetic spectrum and clinical correlates of
413 somatic mutations in aldosterone-producing adenoma. *Hypertension* 2014;64:354-361. doi:
414 10.1161/HYPERTENSIONAHA.114.03419.

415 12) Lenzini L, Rossitto G, Maiolino G, Letizia C, Funder JW, Rossi GP. A Meta-Analysis of Somatic
416 KCNJ5 K(+) Channel Mutations In 1636 Patients With an Aldosterone-Producing Adenoma. *J Clin*
417 *Endocrinol Metab.* 2015;100:E1089-E1095. doi: 10.1210/jc.2015-2149.

418 13) Adams DD, Fastier FN, Howie JB, Kennedy TH, Kilpatrick JA, Stewart RD. Stimulation of the
419 human thyroid by infusions of plasma containing LATS protector. *J Clin Endocrinol Metab.*
420 1974;39:826–832. doi:10.1210/jcem-39-5-826.

421 14) Davies TF, Ando T, Lin RY, Tomer Y, Latif R. Thyrotropin receptor associated diseases: from
422 adenomata to Graves disease. *J Clin Invest.* 2005;115:1972–1983. doi: 10.1172/JCI26031.

423 15) Morshed SA, Davies TF. Graves' Disease Mechanisms: The Role of Stimulating, Blocking, and
424 Cleavage Region TSH Receptor Antibodies. *Horm Metab Res.* 2015;47:727-34.
425 doi: 10.1055/s-0035-1559633.

- 426 16) Wallukat G, Wollenberger A. Effects of the serum gamma globulin fraction of patients with
427 allergic asthma and dilated cardiomyopathy on chronotropic beta adrenoceptor function in
428 cultured neonatal rat heart myocytes. *Biomed Biochim Acta* 1987;46:S634–S639.
- 429 17) Limas CJ, Goldenberg IF, Limas C. Autoantibodies against betaadrenoceptors in human
430 idiopathic dilated cardiomyopathy. *Circ Res.* 1989;64:97–103.
- 431 18) Fu ML, Herlitz H, Wallukat G, Hilme E, Hedner T, Hoebeke J, Hjalmarson A. Functional
432 autoimmune epitope on alpha 1-adrenergic receptors in patients with malignant hypertension.
433 *Lancet* 1994;344:1660–1663.
- 434 19) Luther HP, Homuth V, Wallukat G. Alpha 1-adrenergic receptor antibodies in patients with
435 primary hypertension. *Hypertension* 1997;29:678–682.
- 436 20) Wallukat G, Homuth V, Fischer T, Lindschau C, Horstkamp B, Jüpner A, Baur E, Nissen E, Vetter
437 K, Neichel D, Dudenhausen JW, Haller H, Luft FC. Patients with preeclampsia develop agonistic
438 autoantibodies against the angiotensin AT1 receptor. *J Clin Invest.* 1999;103:945-952
- 439 21) Wenzel K, Haase H, Wallukat G, et al. Potential relevance of alpha(1)-adrenergic receptor
440 autoantibodies in refractory hypertension. *PLoS One* 2008;3:e3742.
441 doi:10.1371/journal.pone.0003742.
- 442 22) Dragun D, Müller DN, Bräsen JH, et al. Angiotensin II type 1-receptor activating antibodies in
443 renal-allograft rejection. *N Engl J Med.* 2005;352:558-569.
- 444 23) Dragun D. Humoral responses directed against non-human leukocyte antigens in solid-organ
445 transplantation. *Transplantation* 2008;86:1019-1025. doi: 10.1097/TP.0b013e3181889748.
- 446 24) Dragun D, Philippe A. From mother to child--transplacental effect of AT1R-AA in preeclampsia.
447 *Nephrol Dial Transplant.* 2010;25:1774-6. doi: 10.1093/ndt/gfq167.
- 448 25) Luft FC. Activating autoantibodies and cardiovascular disease. *Physiology (Bethesda)*
449 2013;28:254-261. doi: 10.1152/physiol.00014.2013.

450 26) Siddiqui AH, Irani RA, Blackwell SC, Ramin SM, Kellems RE, Xia Y. Angiotensin receptor
 451 agonistic autoantibody is highly prevalent in preeclampsia: correlation with disease severity.
 452 *Hypertension* 2010;55:386-393. doi: 10.1161/HYPERTENSIONAHA.109.140061

453 27) Wenzel K, Rajakumar A, Haase H, et al. Angiotensin II type 1 receptor antibodies and increased
 454 angiotensin II sensitivity in pregnant rats. *Hypertension* 2011;58:77-84.
 455 doi: 10.1161/HYPERTENSIONAHA.111.171348.

456 28) Rossitto G, Regolisti G, Rossi E, Negro A, Nicoli D, Casali B, Toniato A, Caroccia B, Seccia TM,
 457 Walther T, Rossi GP. Elevation of angiotensin-II type-1-receptor autoantibodies titer in primary
 458 aldosteronism as a result of aldosterone-producing adenoma. *Hypertension* 2013;61:526-533.
 459 doi: 10.1161/HYPERTENSIONAHA.112.202945

460 29) Kem DC, Li H, Velarde-Miranda C, Liles C, Vanderlinde-Wood M, Galloway A, Khan M, Zillner C,
 461 Benbrook A, Rao V, Gomez-Sanchez CE, Cunningham MW, Yu X. Autoimmune mechanisms
 462 activating the angiotensin AT1 receptor in 'primary' aldosteronism. *J Clin Endocrinol Metab.*
 463 2014;99:1790-1797. doi: 10.1210/jc.2013-3282

464 30) Sabbadin C, Ceccato F, Ragazzi E, Boscaro M, Betterle C, Armanini D. Evaluation of angiotensin
 465 II type-1 receptor antibodies in primary aldosteronism and further considerations about their
 466 possible pathogenetic role. *J Clin Hypertens (Greenwich)*. 2018;20:1313-1318.
 467 doi: 10.1111/jch.13351.

468 31) Funder JW, Carey RM, Mantero F, Murad MH, Reincke M, Shibata H, Stowasser M, Young WF
 469 Jr. The Management of Primary Aldosteronism: Case Detection, Diagnosis, and Treatment: An
 470 Endocrine Society Clinical Practice Guideline. *J Clin Endocrinol Metab.* 2016;101:1889-1916.
 471 doi: 10.1210/jc.2015-4061.

- 472 32) Williams TA, Reincke M. MANAGEMENT OF ENDOCRINE DISEASE: Diagnosis and management
473 of primary aldosteronism: the Endocrine Society guideline 2016 revisited. *Eur J Endocrinol.*
474 2018;179:R19-R29. doi: 10.1530/EJE-17-0990.
- 475 33) Williams B, Mancia G, Spiering W, et al; ESC Scientific Document Group . 2018 ESC/ESH
476 Guidelines for the management of arterial hypertension. *Eur Heart J.* 2018;39:3021-3104.
477 doi: 10.1093/eurheartj/ehy339.
- 478 34) Tranquilli AL, Dekker G, Magee L, Roberts J, Sibai BM, Steyn W, Zeeman GG, Brown MA.
479 The classification, diagnosis and management of the hypertensive disorders of pregnancy: A
480 revised statement from the ISSHP. *Pregnancy Hypertens.* 2014;4:97-104.
481 doi: 10.1016/j.preghy.2014.02.001.
- 482 35) Kahaly GJ, Bartalena L, Hegedüs L, Leenhardt L, Poppe K, Pearce SH. 2018 European Thyroid
483 Association Guideline for the Management of Graves' Hyperthyroidism. *Eur Thyroid J.* 2018;7:167-
484 186. doi: 10.1159/000490384.
- 485 36) Philogene MC, Bagnasco S, Kraus ES, Montgomery RA, Dragun D, Leffell MS, Zachary AA,
486 Jackson AM. Anti-Angiotensin II Type 1 Receptor and Anti-Endothelial Cell Antibodies: A Cross-
487 Sectional Analysis of Pathological Findings in Allograft Biopsies. *Transplantation* 2017;101:608-
488 615. doi: 10.1097/TP.0000000000001231.
- 489 37) Dragun D, Catar R, Philippe A. Non-HLA antibodies against endothelial targets bridging allo-
490 and autoimmunity. *Kidney Int.* 2016;90:280-288. doi: 10.1016/j.kint.2016.03.019.
- 491 38) Lingam RK, Sohaib SA, Vlahos I, Rockall AG, Isidori AM, Monson JP, Grossman A, Reznick RH.
492 CT of primary hyperaldosteronism (Conn's syndrome): the value of measuring the adrenal gland.
493 *AJR Am J Roentgenol.* 2003;181:843-849.
- 494 39) Rieber-Mohn AB, Sugulle M, Wallukat G, Alnæs-Katjavivi P, Leite Størvold G, Bolstad N,
495 Redman CW, Dechend R, Staff AC. Auto-antibodies against the angiotensin II type I receptor in

496 women with uteroplacental acute atherosclerosis and preeclampsia at delivery and several years
 497 postpartum. *J Reprod Immunol*. 2018;128:23-29. doi: 10.1016/j.jri.2018.05.008.

498 40) Zhang S, Zheng R, Yang L, Zhang X, Zuo L, Yang X, Bai K, Song L, Tian J, Yang J, Liu H.
 499 Angiotensin type 1 receptor autoantibody from preeclamptic patients induces human
 500 fetoplacental vasoconstriction. *J Cell Physiol*. 2013;228:142-148. doi: 10.1002/jcp.24113.

501 41) Jahns R, Boege F. Questionable validity of peptide-based ELISA strategies in the diagnostics of
 502 cardiopathogenic autoantibodies that activate G-protein-coupled receptors. *Cardiology*
 503 2015;131:149-150. doi: 10.1159/000376546.

504 42) Meyer LS, Wang X, Sušnik E, et al. Immunohistopathology and steroid profiles associated with
 505 biochemical outcomes after adrenalectomy for unilateral primary aldosteronism. *Hypertension*
 506 2018;72:650-657. doi: 10.1161/HYPERTENSIONAHA.118.11465.

507 43) Omata K, Satoh F, Morimoto R, Ito S, Yamazaki Y, Nakamura Y, Anand SK, Guo Z, Stowasser M,
 508 Sasano H, Tomlins SA, Rainey WE. Cellular and genetic causes of idiopathic hyperaldosteronism.
 509 *Hypertension* 2018;72:874-880. doi: 10.1161/HYPERTENSIONAHA.118.11086.

510 44) Williams TA, Mulatero P, Bidlingmaier M, Beuschlein F, Reincke M. Genetic and potential
 511 autoimmune triggers of primary aldosteronism. *Hypertension* 2015;66:248-253.
 512 doi: 10.1161/HYPERTENSIONAHA.115.05643.

513 45) Maning J, Negussie S, Clark MA, Lymperopoulos A. Biased agonism/antagonism at the AngII-
 514 AT1 receptor: Implications for adrenal aldosterone production and cardiovascular therapy.
 515 *Pharmacol Res*. 2017;125:14-20. doi: 10.1016/j.phrs.2017.05.009.

516 46) Cahill TJ 3rd, Thomsen AR, Tarrasch JT, et al. Distinct conformations of GPCR- β -arrestin
 517 complexes mediate desensitization, signaling, and endocytosis. *Proc Natl Acad Sci U S A*.
 518 2017;114:2562-2567. doi: 10.1073/pnas.1701529114.

- 519 47) Lympelopoulos A, Rengo G, Zincarelli C, Kim J, Koch WJ. Adrenal beta-arrestin 1 inhibition in
520 vivo attenuates post-myocardial infarction progression to heart failure and adverse remodeling via
521 reduction of circulating aldosterone levels. *J Am Coll Cardiol*. 2011;57:356-365.
522 doi: 10.1016/j.jacc.2010.08.635.
- 523 48) Valero TR, Sturchler E, Jafferjee M, Rengo G, Magafa V, Cordopatis P, McDonald P, Koch WJ,
524 Lympelopoulos A. Structure-activity relationship study of angiotensin II analogs in terms of β -
525 arrestin-dependent signaling to aldosterone production. *Pharmacol Res Perspect*. 2016;4:e00226.
526 doi: 10.1002/prp2.226.
- 527 49) Schulz A, Jankowski J, Zidek W, Jankowski V. Absolute quantification of endogenous
528 angiotensin II levels in human plasma using ESI-LC-MS/MS. *Clin Proteomics* 2014;11:37.
529 doi: 10.1186/1559-0275-11-37. eCollection 2014.
- 530 50) Gomez-Sanchez CE, Kuppusamy M, Reincke M, Williams TA. Disordered CYP11B2 Expression in
531 Primary Aldosteronism. *Horm Metab Res*. 2017;49:957-962. doi: 10.1055/s-0043-122238.

Novelty and Significance

What is New?

- AT1R-Ab levels were measured in groups of patients with hypertension compared with normotensive individuals
- Higher agonistic AT1R-Abs levels were present in bilateral primary aldosteronism, primary hypertension and preeclampsia groups compared with normotensive individuals
- Patients with bilateral *versus* unilateral primary aldosteronism had higher levels of agonistic AT1R-Abs

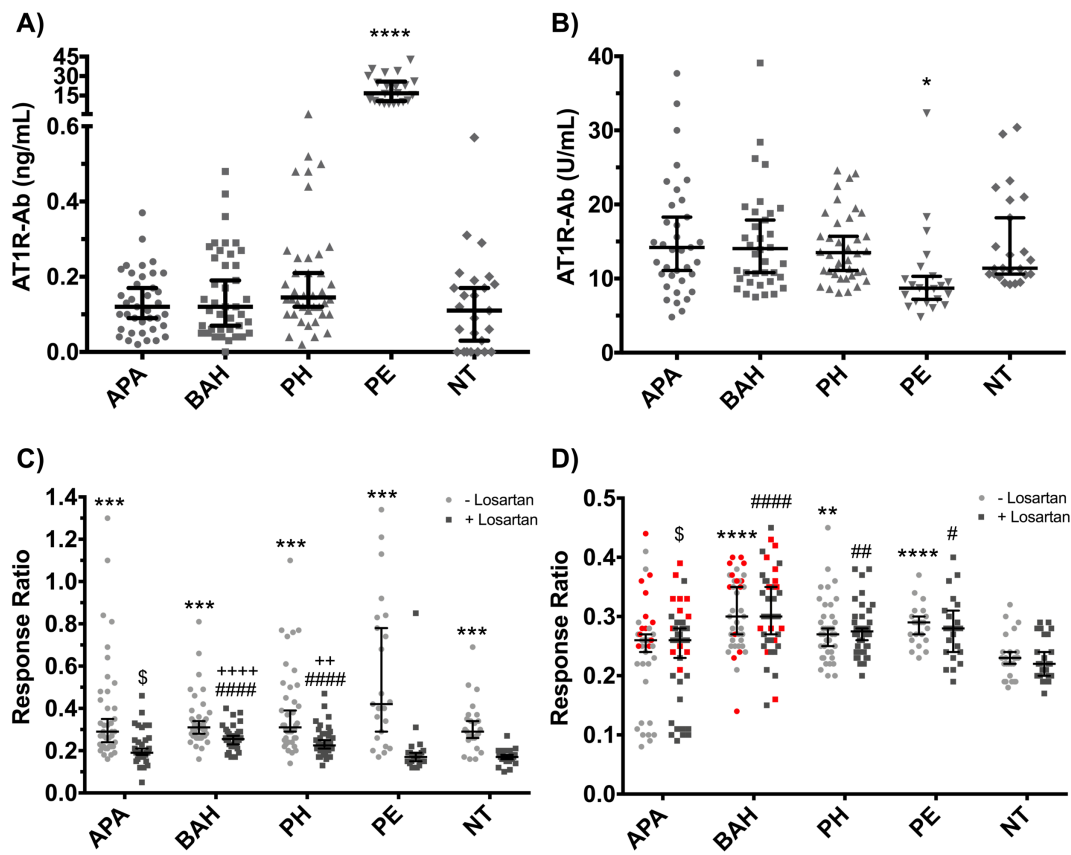
What is relevant?

- AT1R-Abs measured by ELISA did not correlate with functional activation of the AT1R
- Patients with higher AT1R-Ab activity levels have an increased likelihood of a diagnosis of bilateral than unilateral primary aldosteronism
- Higher levels of agonistic AT1R-Abs were associated with higher aldosterone-to-renin ratios and lower plasma renin concentrations
- Patients with primary aldosteronism with adrenal hyperplasia displayed higher agonistic AT1R-Abs levels

Summary

Agonistic autoantibodies to the AT1R are present in patients with disorders related to hypertension and may contribute to autonomous aldosterone production and adrenal hyperplasia in a subgroup of patients with primary aldosteronism

Figure Legend



Measurement of AT1R autoantibodies and AT1R activating response in patients with primary aldosteronism, primary hypertension, preeclampsia and in normotensive individuals

Scatter dot plots showing quantification of AT1R-Ab in total serum of patients with PA (APA and BAH), PH, PE and normotensive individuals by measurements using ELISA-Creative Diagnostics (**Panel A**) or ELISA-CellTrend (**Panel B**). A cell-based AT1R activation assay was used to measure AT1R-Ab agonist activity in total serum (**Panel C**) or in agarose-A/G affinity isolated IgG fractions (**Panel D**) in the absence (**light grey points**) or presence (**dark grey points**) of 100 μ m losartan as indicated. **Panel D** also highlights the agonistic AT1R-Ab levels in patients with adrenal hyperplasia at CT imaging (**red points**). The response ratio represents AT1R-activation of β -lactamase activity measured as coumarin to fluorescein fluorescence (cleaved to uncleaved substrate ratio) normalized for negative controls. Horizontal lines within boxes indicate the median, and the lower and upper horizontal lines indicate the 95% CI. *P* values were calculated using the Kruskal-Wallis test and indicate **** difference ($P < 0.0001$) from NT (**Panel A**); * difference ($P < 0.05$) from NT

(**Panel B**); *** difference ($P<0.001$) absence *versus* presence of losartan for each subgroup; \$ difference ($P<0.01$) from BAH; #### difference ($P<0.0001$) from NT (presence of losartan); ++++ difference ($P<0.0001$) from PE (presence of losartan); ++ difference ($P<0.01$) from PE (presence of losartan); (**Panel C**); ** difference ($P<0.01$) from NT (absence of losartan), **** difference ($P<0.0001$) from NT (absence of losartan); \$ difference ($P<0.01$) (presence of losartan); #### difference ($P<0.0001$) from NT (presence of losartan); ## difference ($P<0.01$) from NT (presence of losartan); # difference ($P<0.05$) from NT (presence of losartan); (**Panel D**). Numbers of patient samples in each subgroup were APA, $N=40$; BAH, $N=40$; PH, $N=40$; PE, $N=23$; NT, $N=25$. APA, aldosterone-producing adenoma; AT1R-Ab, angiotensin II type 1 receptor autoantibodies; BAH, bilateral adrenal hyperplasia; PH, primary hypertension; PE, preeclampsia; NT, normotensive individuals.

Clinical parameter	APA	BAH	PH	Overall	Pairwise comparisons		
	(N=40)	(N=40)	(N=40)	P-value	APA vs BAH	APA vs PH	BAH vs PH
Age (years)	52 ± 10.2	52 ± 9.7	52 ± 19.9	0.964	N.A.	N.A.	N.A.
Sex (ref. male)	21 (52.5%)	19 (47.5%)	16 (42.1%)	0.656	N.A.	N.A.	N.A.
BMI (Kg/m ²)	27.3 ± 4.1	26.2 ± 5.0	27.4 ± 6.0	0.500	N.A.	N.A.	N.A.
Systolic BP (mmHg)	151 ± 21.5	151 ± 23.8	156 ± 17.2	0.461	N.A.	N.A.	N.A.
Diastolic BP (mmHg)	93 ± 11.0	95 ± 13.6	91 ± 14.6	0.469	N.A.	N.A.	N.A.
PAC (pmol/L)	569 [283-1071]	416 [311-583]	225 [128-394]	< 0.001	0.742	< 0.001	0.002
DRC (mU/L)	4.3 [2.0-11.2]	3.4 [2.0-7.3]	18.2 [8.9-45.1]	< 0.001	0.831	< 0.001	< 0.001
ARR_DRC	108 [36-306]	114 [71-162]	16 [6-26]	< 0.001	1.000	< 0.001	< 0.001
Lowest serum K ⁺ (mmol/L)	2.9 [2.6-3.2]	3.3 [3.0-3.7]	3.9 [3.6-4.2]	< 0.001	0.001	< 0.001	< 0.001

Table 1. Clinical parameters of patients with primary aldosteronism and primary hypertension

Clinical data of patients with PA (APA or BAH) and PH are presented as average values ± SD, absolute numbers with proportions in parenthesis (%) or as medians with lower and upper quartiles in parentheses. *P* values designate the presence of group differences by the ANOVA and Bonferroni post-hoc tests (age, BMI, systolic and diastolic BP), Kruskal–Wallis test (PAC, DRC, ARR_DRC and potassium), or Chi square test (sex). Numbers of patient samples in each subgroup are indicated. APA, aldosterone-producing adenoma; ARR_DRC, aldosterone-to-renin ratio using direct renin

measurements; BAH, bilateral adrenal hyperplasia; BMI, body mass index; BP, blood pressure; DRC, direct renin concentration; PAC, plasma aldosterone concentration; PH, primary hypertension.

Clinical parameter	AT1R-Ab level minus losartan		<i>P</i> -value	AT1R-Ab level plus losartan		<i>P</i> -value
	< median	≥ median		< median	≥ median	
Diagnosis: APA	24 (46.2)	16 (23.5)	0.009	23 (40.4)	17 (27.0)	0.120
BAH	12 (23.1)	28 (41.2)	0.037	14 (24.6)	26 (41.3)	0.053
PH	16 (30.7)	24 (35.3)	0.603	20 (35.1)	20 (31.7)	0.699
Age (years)	54 ± 14.8	55 ± 16.6	0.749	54 ± 15.5	55 ± 16.2	0.851
Sex (ref. male)	30 (57.7)	39 (57.4)	0.970	28 (49.1)	41 (65.1)	0.077
BMI (Kg/m ²)	28.2 ± 4.7	27.5 ± 5.0	0.431	27.2 ± 4.2	28.4 ± 5.3	0.177
Systolic BP (mmHg)	151 ± 23.9	147 ± 19.2	0.376	148 ± 25.1	149 ± 17.4	0.709
Diastolic BP (mmHg)	92 ± 15.0	86 ± 12.5	0.018	89 ± 16.9	89 ± 10.7	0.854
PAC (pmol/L)	235 [150-553]	300 [167-556]	0.499	236 [130-550]	286 [186-569]	0.338
DRC (mU/L)	11.7 [5.7-31.8]	5.7 [2.2-27.0]	0.011	11.9 [5.3-39.7]	5.6 [2.3-16.3]	0.003
ARR_DRC	23 [10-55]	47 [13-139]	0.029	19 [7-60]	49 [16-137]	0.003
Lowest serum K ⁺ (mmol/L)	3.2 [2.9-3.9]	3.4 [3.2-3.9]	0.333	3.3 [2.9-3.9]	3.4 [3.2-3.9]	0.084

Table 2. Clinical parameters of patients with primary aldosteronism and primary hypertension according to functional AT1R-Ab levels

Clinical parameters of the combined cohort of patients with APA, BAH and PH were analyzed according to AT1R-Ab levels (affinity-purified autoantibody activity measured with the cell-based assay) categorized according to the median value of the combined cohort (median values, 0.27 and 0.28 in the absence and presence of losartan respectively). Data are presented as average values \pm SD, absolute numbers with proportions in parenthesis (%) or as medians with lower and upper quartiles in parentheses. *P* values designate the presence of group differences by the ANOVA and Bonferroni post-hoc tests (age, BMI, systolic and diastolic BP), Kruskal–Wallis test (PAC, DRC, ARR_DRC and potassium), or Chi square test (sex, diagnosis). Numbers of patient samples in each subgroup are indicated. APA, aldosterone-producing adenoma; ARR_DRC, aldosterone-to-renin ratio using direct renin measurements; BAH, bilateral adrenal hyperplasia; BMI, body mass index; BP, blood pressure; DRC, direct renin concentration; PAC, plasma aldosterone concentration; PH, primary hypertension.

Clinical parameter	BAH vs. APA		BAH vs. PH	
	OR (CI 95%)	P-value	OR (CI 95%)	P-value
Agonistic AT1R-Ab level - losartan				
AT1R-Abs (ref. ≥ median)	3.425 (1.342-8.696)	0.010	1.515 (0.589-3.891)	0.388
Age (years)	0.976 (0.941-1.012)	0.186	1.025 (0.997-1.053)	0.078
AT1R-Abs (ref. ≥ median)	3.663 (1.420-9.434)	0.007	1.495 (0.587-8.817)	0.339
Systolic BP (mmHg)	1.019 (0.005-1.044)	0.116	0.993 (0.972-1.015)	0.532
AT1R-Abs (ref. ≥ median)	3.521 (1.361-9.091)	0.009	1.887 (0.688-5.319)	0.231
PAC (pmol/L)	1.001 (1.000-1.003)	0.072	1.003 (1.001-1.005)	0.003
AT1R-Abs (ref. ≥ median)	3.546 (1.395-9.009)	0.008	1.603 (0.630-4.065)	0.322
DRC (mU/L)	0.996 (0.989-1.004)	0.298	0.996 (0.990-1.002)	0.221
Agonistic AT1R-Ab level + losartan				
AT1R-Abs (ref. ≥ median)	2.571 (1.027-6.452)	0.044	1.980 (0.786-5.000)	0.147
Age (years)	0.973 (0.938-1.009)	0.135	1.026 (0.999-1.055)	0.062
AT1R-Abs (ref. ≥ median)	2.358 (0.943-5.882)	0.066	1.832 (0.745-4.505)	0.187
Systolic BP (mmHg)	1.015 (0.992-1.039)	0.211	0.993 (0.971-1.014)	0.497
AT1R-Abs (ref. ≥ median)	2.381 (0.947-5.988)	0.065	2.278 (0.838-6.211)	0.107
PAC (pmol/L)	1.001 (1.000-1.002)	0.086	1.003 (1.001-1.005)	0.003
AT1R activation (ref. ≥ median)	2.500 (1.007-6.211)	0.048	1.698 (0.678-4.255)	0.258
DRC (mU/L)	0.966 (0.989-1.004)	0.323	0.997 (0.990-1.003)	0.314

Table 3. Association of agonistic affinity-purified AT1R-Ab levels and diagnosis of BAH

Logistic regression analyses were performed to determine the potential association of agonistic autoantibody levels with a diagnosis of BAH with adjustment for confounding effects of a single clinical variable per level (age, systolic BP, PAC or DRC) in the absence and presence of losartan. Autoantibody levels were categorized according to the median affinity-purified AT1R-Ab level in the cell-based assay as shown. Data are presented as odds ratios (OR) with 95% confidence intervals (CI). An OR > 1 indicates an increased likelihood for a diagnosis of BAH in the presence of agonistic AT1R-Ab activity \geq median value independent of the tested confounding variable (age, systolic BP, PAC, DRC). APA, aldosterone-producing adenoma; AT1R, angiotensin II type 1 receptor; BAH, bilateral adrenal hyperplasia; BP, blood pressure; DRC, direct renin concentration; PAC, plasma aldosterone concentration; PH, primary hypertension; ref, reference.

Clinical parameter	Hyperplasia		<i>P</i> -value
	Absence (n=35)	Presence (n=25)	
Diagnosis			
APA	25 (71.4)	12 (48.0)	0.066
BAH	10 (28.6)	13 (52.0)	
Agonistic AT1R-Ab level - losartan			
AT1R-Abs (response ratio)	0.26 [0.23-0.29]	0.30 [0.26-0.39]	0.011
AT1R-Abs (ref. \geq median)	13 (37.1)	19 (76.0)	0.003
Agonistic AT1R-Ab level + losartan			
AT1R-Abs (response ratio)	0.27 [0.20-0.30]	0.30 [0.24-0.36]	0.149
AT1R-Abs (ref. \geq median)	16 (45.7)	15 (60.0)	0.205

Table 4. Functional AT1R autoantibody levels stratified by adrenal morphology

Adrenal morphology of patients with PA was determined from CT results to classify absence or presence of hyperplasia in adrenals with morphologic abnormalities. Numbers of patient samples in each subgroup are indicated. Affinity-purified agonistic AT1R-Ab levels, measured with the cell-based assay, were treated as continuous variables and presented as medians with lower and upper quartiles in parenthesis or categorized as higher and lower agonistic AT1R-Ab levels according to the median value for patients with APA and BAH combined and presented as absolute numbers with proportions in parenthesis. *P* values designate the presence of group differences by the Kruskal–Wallis test (AT1R-Ab levels), or Chi square test (diagnosis, AT1R-Ab levels after categorization).